REMARKS

The Office action mailed July 29, 2003, has been reviewed and carefully considered. Claim 12 has been amended to correct punctuation. The specification has been amended to include a reference to the priority application. Claims 1, 3-5, 12, 14 and 16-26 were pending in the application. Claims 27 and 28 have been added. Support for claims 27 and 28 is found in the specification, for example, at page 5, lines 3-4. Therefore, claims 1, 3-5, 12, 14 and 16-28 remain pending.

Applicants thank the Examiner for withdrawing the previous rejections under the judicially created doctrine of obviousness-type double patenting and under 35 U.S.C. § 102(e).

All of the pending claims were rejected under 35 U.S.C. § 103(a) as unpatentable over U.S. Patent No. 5,738,855 (herein the '855 patent). Applicants respectfully disagree and request reconsideration.

It is asserted in the Office action on page 4, second paragraph, that "it would have been obvious ... to modify the Vi-rEPA conjugate of [the '855 patent] that comprised a cystamine linker with the adipic dihydrazide linker." Page 5 of the Office action goes on to state that "[i]n the absence of a showing of unexpected results, [the '855 patent] obviates the instantly claimed invention."

Applicants submit that the application itself includes such evidence of unexpected results.[1] Page 5, lines 4-8, of the present application states that "the exemplified Vi-*r*EPA conjugate vaccine of this invention using ADH as the linker (i.e., Vi-*r*EPA_{II}) is capable of inducing serum IgG antibody levels which are statistically significantly higher than those induced by Vi alone or by Vi conjugated to *r*EPA using SPDP as the linker (i.e., Vi-*r*EPA_{I})." As explained below in more detail, the results in the application directly compare a vaccine with an

[1] Such evidence must be considered according to MPEP §716.01(a); See also, *In re Soni*, 34 USPQ2d 1684, 1687 (Fed. Cir. 1995) ("the PTO must consider comparative data in the specification in determining whether the claimed invention provides unexpected results").

ADH linker versus a vaccine with a cystamine/SPDP linker system as described in the '855 patent.

Example 3 of the '855 patent describes the synthesis of the only exemplified conjugate. In Example 3, Vi was derivatized with cystamine via a 1-ethyl-3(d-dimethylaminopropyl) carbodimide-mediated reaction (see column 11, lines 33-47). EPA was derivatized with SPDP via an EDTA-mediated reaction (see column 11, lines 48-58). The Vi-*r*EPA conjugate then was formed by mixing together the cystamine-derivatized Vi and the SPDP-derivatized EPA (see column 11, lines 60-67). The resulting conjugate included a cystamine/SPDP linker system.

The comparative example (Vi-*r*EPA_I) described on page 13 of the present application is prepared via the same synthetic route as described in Example 3 of the '855 patent. Vi was derivatized with cystamine via a 1-ethyl-3(d-dimethylaminopropyl) carbodimide-mediated reaction (see page 13, lines 3-9). EPA was derivatized with SPDP via an EDTA-mediated reaction (see page 13, lines 10-15). The Vi-*r*EPA conjugate then was formed by mixing together the cystamine-derivatized Vi and the SPDP-derivatized EPA (see page 13, lines 16-25). The resulting Vi-*r*EPA_I conjugate included a cystamine/SPDP linker system, which is the same as the linker system employed in Example 3 of the '855 patent.

An example of the presently claimed vaccine (Vi-*r*EPA_{II}) is described on pages 13-14 of the present application. The Vi-*r*EPA_{II} conjugate includes the same S. *typhi* Vi polysaccharide and carrier protein *r*EPA as the Vi-*r*EPA_I conjugate and the Vi-*r*EPA of the '855 patent. The difference is that the Vi-*r*EPA_{II} conjugate includes an adipic acid dihyrazide (ADH) linker while the Vi-*r*EPA_I conjugate and the Vi-*r*EPA of the '855 patent both include a cystamine/SPDP linker system. Hence, a comparison between Vi-*r*EPA_I and Vi-*r*EPA_{II} is a direct comparison between an ADH linker and a cystamine/SPDP linker system as suggested by the examiner in the Office action.

Tables 5 and 6 of the present application provide comparative clinical data in children demonstrating that the ADH linker provides an unexpectedly superior result relative to the cystamine/SPDP linker system. As shown in Table 5, a single injection of Vi-*r*EPA_{II} elicited

higher levels of IgG than did a single injection of Vi-*r*EPA$_I$. For example, at 26 weeks, IgG levels using Vi-*r*EPA$_{II}$ were about 3-fold higher than vaccination with Vi-*r*EPA$_I$ (30.0 versus 10.8). As shown in Table 6, two injections of Vi-*r*EPA$_{II}$ elicited higher levels of IgG than did two injections of Vi-*r*EPA$_I$. For example, at 26 weeks, IgG levels using Vi-*r*EPA$_{II}$ were about 2-fold higher than vaccination with Vi-*r*EPA$_I$ (30.6 versus 12.8). In the context of these results, applicants note that "[a]ntibodies of the IgG class are preferred for purposes of passive protection" (present application, page 9, lines 19-20).

Attached as Exhibit A is an article (Szu *et al.*, *Infection and Immunity*, 62:4440-4, 1994). Szu *et al.* relates clinical antibody results for vaccine conjugates made from Vi and *r*EPA with a cystamine/SPDP linker system (see page 4441). Table 4 of the present application and Table 4 of Szu *et al.* (Exhibit A) provide comparative data in adults demonstrating that the ADH linker provides an unexpectedly superior result compared to the cystamine/SPDP linker system. Table 4 of Szu *et al.* provides the results of immunizing adults with the Vi-*r*EPA (cystamine/SPDP linker) conjugate, and Table 4 of the present application provides the results of immunizing adults with the Vi-*r*EPA$_{II}$ (ADH linker) conjugate. As shown in Table 4 of Szu *et al.*, two injections of Vi-*r*EPA (cystamine/SPDP linker) elicited a total antibody level of 2.69 μg/ml 26 weeks after the first injection. As shown in Table 4 of the present application, one injection of Vi-*r*EPA$_{II}$ (ADH linker) elicited an IgG antibody level of 119 μg/ml 26 weeks after the injection (measured via ELISA). Approximately 10 ELISA units of IgG as measured in the present application is equal to one μg/ml of total antibodies as measured in Szu *et al* . Hence, IgG levels using Vi-*r*EPA$_{II}$ (ADH linker) were about 4-fold higher than vaccination with Vi-*r*EPA (cystamine/SPDP linker) (11.9 versus 2.69).

The '855 patent suggests no preference for an ADH linker over any of the other mentioned possible linkers. The only linker exemplified in the '855 patent is cystamine/SPDP. However, it is apparent from the comparative results in the present application that Vi-*r*EPA conjugates with an ADH linker provide unexpectedly superior antibody levels compared to Vi-*r*EPA conjugates with a cystamine/SPDP linker system. Such unexpected results clearly sufficiently rebut any case of *prima facie* obviousness over Szu *et al.*

It is respectfully submitted that the present claims are in condition for allowance. Should there be any questions regarding this application, Examiner Portner is invited to telephone the undersigned attorney at the telephone number shown below.

Respectfully submitted,

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Laboratory and Preliminary Clinical Characterization of Vi Capsular Polysaccharide-Protein Conjugate Vaccines

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To improve its immunogenicity for children and adults and to make it suitable for routine immunization of infants against typhoid fever, the capsular polysaccharide of *Salmonella typhi* (Vi) was bound to the B subunit of the heat-labile toxin (LT-B) of *Escherichia coli* or the recombinant exoprotein A (rEPA) of *Pseudomonas aeruginosa*. The conjugates elicited higher levels of antibodies (micrograms per milliliter of serum) in mice and in guinea pigs than did Vi and, unlike Vi alone, elicited booster antibody responses in both species. In adult volunteers, Vi–LT-B and Vi-rEPA, respectively, elicited higher levels of antibodies than Vi alone after the first injection (4.74 versus 1.77 and 4.91 versus 1.77; $P < 0.005$) and 26 weeks later (2.32 and 2.69 versus 0.54; $P < 0.04$); a second injection of the conjugates did not elicit a booster response of Vi antibodies. None of the 51 vaccinees had fever or significant local reactions. Vi-rEPA elicited slightly higher levels of Vi antibodies than did Vi–LT-B at all intervals after injection, but these differences were not significant. Each conjugate elicited antibodies to its carrier protein. The antibody responses elicited in adults by Vi bound to LT-B and rEPA are similar to those of other polysaccharide-protein conjugates. These conjugates promise to be an improved Vi vaccine. Studies of Vi conjugates with adults and infants in areas where typhoid is endemic are planned.

Typhoid fever remains a common and serious disease in many parts of the world (11, 33). Treatment of typhoid fever has become more difficult with the emergence of antibiotic-resistant strains of *Salmonella typhi* (2, 4, 43). The development of a capsular polysaccharide (Vi) of *S. typhi* provided a simple and safe method for prevention of typhoid fever (26, 32). In two randomized, double-blind, placebo-controlled studies in areas where the disease is highly endemic, one injection of Vi antigen conferred 65 to 70% protection against typhoid on individuals 5 to 45 years of age (1, 24). The usefulness of the Vi vaccine, as has been shown for other capsular polysaccharides (31), is limited by its age-related immunogenicity and its T-cell-independent properties; i.e., it does not elicit protective levels of antibodies in infants and young children, and reinjection at any age does not induce a booster effect (25). These limitations restrict Vi from the routine immunization program for infants and children. Our objectives are to provide a vaccine for prevention of typhoid fever that is more effective than Vi alone in older children and adults and can be administered along with other vaccines used for routine immunization of infants.

To improve its immunologic properties, we covalently bound Vi to cholera toxin (40). The conjugation procedure reduced, but did not eliminate, its toxicity. Later, we used tetanus toxoid (TT) as the carrier protein (Vi-TT). Technical problems were encountered because Vi has an unusually large molecular size and a high level structural rigidity, which resulted in low yields and poor solubility of Vi-TT (39). One preparation of Vi-TT depolymerized during storage and was poorly immunogenic in adults (21a). To circumvent these problems, the molecular size of Vi was reduced by ultrasonic irradiation and it was bound to each of several proteins (38, 41). Although this procedure provided higher yields with improved solubility, the resultant conjugates elicited lower levels of antibodies than did those prepared with untreated Vi.

In the experiments reported here, two proteins of medical interest with lower molecular weights than TT, the B subunit of the heat-labile cholera-like enterotoxin (LT-B) of *Escherichia coli* and the recombinant exoprotein A (rEPA) of *Pseudomonas aeruginosa*, were used as carriers for Vi (7, 8, 14, 15). The immunogenicity of these conjugates in young mice and guinea pigs and their safety in adult volunteers are described.

MATERIALS AND METHODS

Study design. Volunteers between 18 and 44 years of age who had no antibodies to hepatitis B virus and human immunodeficiency virus type 1 were recruited at two institutions. Following receipt of their informed consent, volunteers received one injection of Vi (25 μg in 0.5 ml) (1, 24) or two injections of a conjugate (15 μg in 1.0 ml). Oral temperatures were taken, and the injection site of each volunteer was inspected 6, 24, and 48 h after each injection. Volunteers were bled on day 7 following the first injection for determination of levels of serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase (15). At the first institution, volunteers (16 to 18 for each vaccine) received their second conjugate injection at 4 weeks and were bled 2 weeks later. At the second institution, the volunteers (six to nine for each vaccine) were reinjected with the conjugates at 6 weeks following the first injection and bled 2 weeks later. All volunteers were bled 26 weeks after the first injection. Only 2 of 25 volunteers who received Vi had a third bleeding, and accord-

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TABLE 1. Composition of Vi polysaccharide conjugate vaccines

Conjugate (lot[a] no.)	SH/Vi ratio (% [wt/wt])	SPDP/protein ratio (mol/mol)	Vi concn (μg/ml)	Protein concn (μg/ml)	Protein/Vi ratio (wt/wt)
Vi–LT-B (50860)	1.3	3.5	30	21	0.7
Vi-rEPA (51706)	1.3	4.3	16	81	5.1

[a] One lot of cystamine-derivative Vi was used for the conjugates.

ingly, these values were omitted from the calculations. Since there were no statistically significant differences between the levels of geometric mean antibodies to Vi, LT-B, and rEPA elicited by each vaccine in the two groups, the values were combined.

Reagents. Pyrogen-free water and pyrogen-free saline (PFS) for clinical use were from Baxter, Deerfield, Wis.; cystamine was from Fluka, Ronkonkoma, N.Y.; *N*-succinimidyl-3-(2-pyridythio)propionate (SPDP) was from Pierce Chemical, Rockford, Ill.; NaOH and HCl were from Baker Chemical, Phillipsburg, N.J.; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, dithiothreitol, EDTA, and Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] were from Sigma, St. Louis, Mo.; bicinchoninic acid protein reagent, Sephacryl S-1000, Sephadex G-50, and Superose 6 were from Pharmacia, Piscataway, N.J.; acridine orange was from National Aniline, Chicago, Ill.; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was from Calbiochem, La Jolla, Calif.; and BioGel P10 was from Bio-Rad, Richmond, Calif.

Antigens. The Vi (lot 104A) purified from *S. typhi* Ty-2 (a gift from Pasteur Mérieux, Lyon, France) conformed to the requirements of the World Health Organization (46). The B subunit of *E. coli* LT-B, lot 84019, was purified from strain JM83 by affinity chromatography as previously described (7, 8). *P. aeruginosa* rEPA was purified from a recombinant strain of *E. coli* BL21 (IDE3) as previously described (7, 15). *P. aeruginosa* exotoxin A (ETA) was from List Biologics.

Analytical methods. The molecular sizes of the Vi antigen and the conjugates were analyzed with a Superose 6 high-pressure liquid chromatography column in 0.02 M sodium phosphate–0.1 M Na_2SO_4 (pH 7.0). Protein was determined by the bicinchoninic acid protein reagent with bovine serum albumin as the standard. SH groups were measured by the method of Ellman (12), and the Vi content of the conjugates was measured by acridine orange binding (37).

The toxicity of LT-B alone and as a conjugate was assayed with CHO cells (13). rEPA and Vi-rEPA (lot 51706) were assayed for ETA activity by injection into mice and recording of survival and serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase levels 48 h later (15).

Synthesis of Vi conjugates. Conjugation of Vi antigen to proteins has already been described (5, 38, 40).

SPDP derivatization of proteins. LT-B and rEPA were reconstituted in PFS to 10 mg/ml and passed through a column (2.5 by 30 cm) Sephadex G-25 in PFS–75 mM HEPES–0.5 M EDTA (pH 6.9) (HEPES buffer). SPDP (40 mM in absolute ethanol) was added dropwise to a final SPDP/LT-B molar ratio of 16:1 and to a final SPDP/rEPA molar ratio of 20:1. The reaction was carried out at room temperature for 1 h, and the mixture was dialyzed against HEPES buffer overnight. The reaction mixture was passed through a column (2.5 by 30 cm) of Sephadex G-25 in HEPES buffer. The protein-containing fractions were pooled and sterilely filtered. The SPDP/protein molar ratios were 3.5 for LT-B and 4.3 for rEPA.

Derivatization of Vi and conjugation to SPDP proteins. Vi (5 mg/ml) was dissolved in 0.2 M NaCl. Cystamine (0.1 M) was mixed with Vi, and the pH was adjusted to 5.0. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.1 M) was added at 37°C, and the reaction mixture was stirred for 4 h. The reaction mixture was dialyzed at 3 to 8°C against PFS with 0.01 M phosphate buffer at pH 7 for 24 h and then dialyzed repeatedly against distilled water and freeze-dried. The cystamine-derivatized Vi was dissolved in PFS and reduced with 50 mM dithiothreitol at room temperature for 2 h. The reaction mixture was passed through a column (2.5 by 30 cm) of Bio-Gel P10 in PFS with 10 mM phosphate buffer at pH 7, and void volume fractions were pooled, mixed with one of the SPDP-derivatized proteins, and stirred slowly at room temperature under argon. The reaction mixture was passed through a column (2.5 by 90 cm) of Sephacryl S-1000 in PFS, and the void volume fractions were delivered to 5.0-ml vials.

Immunization of mice and guinea pigs. Female BALB/c or general-purpose mice from the National Institutes of Health colony (16 to 20 g; 10 in each group) were injected on days 0, 14, and 21 and exsanguinated on days 7, 21, and 28. Guinea pigs (6 weeks old) were injected subcutaneously on days 0, 21, and 42 with 5 μg of Vi alone or as a conjugate (assay performed by Pasteur-Mérieux, Lyon, France). The guinea pigs were bled on days 0, 21, 42, and 64. Groups of four were injected with each vaccine, and the sera from each bleeding were pooled.

Serology. Immunodiffusion was performed in 1% agarose in phosphate-buffered saline (pH 7.2) with hyperimmune burro serum (B260) against *S. typhi* Ty-2 (30) and burro cholera toxin antiserum (B241) (10). Rocket immunoelectrophoresis was performed as previously described (3), with B241 and goat ETA antiserum in the intermediate gels. Vi antibodies in serum were measured by radioimmunoassay (RIA) (42). The sensitivity of this assay was 0.05 μg of antibody per ml. Antibodies to LT-B and ETA in serum were measured by

TABLE 2. Vi antibody levels in sera of female mice injected subcutaneously with Vi alone or as a conjugate[a]

Mice and vaccine (lot no.)	GM antibody concn[b] (μg/ml)		
	1st injection	2nd injection	3rd injection
General purpose (NIH)			
Vi (104a)	1.37	ND	ND
Vi–LT-B (50860)	6.26	12.3	ND
Vi-rEPA (51706)	8.82	59.7	75.2
BALB/c			
Vi (104a)	1.06	1.31	ND
Vi–LT-B (50860)	3.62	6.87	ND
Vi-rEPA (51706)	0.85	17.1	12.7

[a] Mice (20 g; 10 in each group) were injected subcutaneously with saline or with 0.25 μg of Vi alone or as a conjugate on days 0, 14, and 21 and bled on days 7, 21, and 28. Vi antibodies were measured by RIA (41). Mice injected with saline had undetectable levels of Vi antibodies (data not shown).

[b] 6.26 and 8.82 versus 1.37, $P = <0.001$; 3.62 versus 1.06, $P = 0.0009$; 6.87 and 17.1 versus 1.31, $P = 0.0001$. ND, not done.

TABLE 3. Vi antibody levels in sera of female six-week-old guinea pigs injected subcutaneously with Vi alone or as a conjugate[a]

Vaccine (lot no.)	Immunoglobulin G Vi antibodies (ELISA units) in serum		
	1st injection	2nd injection	3rd injection
Vi	<2	<2	<2
Vi–LT-B (50860)	<2	18	ND[b]
Vi-rEPA (51706)	31	384	167

[a] Groups of four guinea pigs, injected subcutaneously with 5.0 μg of Vi alone or as a conjugate on days 0, 21, and 42, were bled on days 0, 21, 42, and 64. Their sera were pooled and assayed for immunoglobulin G Vi antibodies by ELISA. Titers are expressed as ELISA units referable to a standard serum arbitrarily assigned a value of 100 U.

[b] ND, not done.

enzyme-linked immunosorbent assay (ELISA) (15, 38). For each protein, serum from a volunteer with a high titer was chosen as a reference and assigned a value of 100 U. The results are expressed as ELISA units referable to this serum.

Statistics. All calculations used logarithms of antibody concentrations. Each concentration below the sensitivity of the RIA or the ELISA was assigned one-half of that value. Comparisons of GMs were performed by paired and unpaired *t* tests when appropriate. Data were analyzed with the Statistical Analysis System.

RESULTS

Composition of conjugates (Table 1). One lot of a cystamine derivative of Vi antigen (1.3%, wt/wt) was used for both conjugates. The molar ratios of derivatization with SPDP were 3.5 for LT-B and 4.3 for rEPA. Both conjugates passed through the void volume of a Sephacryl S-1000 column, and both failed to enter a 0.8% agarose gel for double immunodiffusion analysis. In rocket immunoelectrophoresis, the Vi and the proteins migrated as one species as detected with rabbit antisera to ETA or to LT-B in the intermediate gels and antiserum to Vi in the running gels (data not shown). The protein/Vi ratios (wt/wt) were 0.7 for Vi–LT-B (lot 50860) and 5.1 for Vi-rEPA (lot 51760).

Neither LT-B (100 μg/ml) nor Vi–LT-B (21 μg of protein per ml) showed toxicity in the CHO cell assay. Similarly, neither 100 μg of rEPA alone or Vi-rEPA (25 μg of protein) caused death or elevated the serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase levels of the mice (these assays can detect 0.1 μg of ETA). A human dose of either conjugate did not induce fever in the rabbit pyrogen assay, and each passed the toxicity tests of mice and guinea pigs prescribed in the *U.S. Code of Federal Regulations* (600:16).

Serum Vi antibodies in mice (Table 2). As expected, Vi alone elicited antibodies in both general-purpose and BALB/c mice (26). Vi–LT-B elicited a significant rise in Vi antibodies (micrograms per milliliter of serum) after the first and second injections in general-purpose (6.26 and 12.3 versus <0.03; $P = 0.0001$) and BALB/c (3.62 and 6.87 versus <0.03; $P = 0.0001$) mice. The second injection of Vi–LT-B elicited booster responses in general-purpose mice (12.3 versus 6.26; $P = 0.03$), but the rise in BALB/c mice (6.87 versus 3.62) was not statistically significant. Vi-rEPA elicited significant rises in Vi antibodies in general-purpose mice after the first (8.82 versus <0.03; $P = 0.0001$) and second (59.7 versus 8.82; $P = 0.0001$) injections. The Vi antibody level rose to 75.2 μg/ml after the third injection, but this level was not significantly different from 59.7 μg/ml. In BALB/c mice, Vi-rEPA elicited a level of antibodies similar to that elicited by Vi after the first injection. The second injection of Vi-rEPA elicited a significant rise (17.1 versus 0.85; $P = 0.0001$). The level of Vi antibodies declined to 12.7 μg/ml after the third injection, but this level did not differ significantly from 17.1 μg/ml.

After the first injection, conjugate-induced levels of antibodies were higher than those elicited by Vi alone in general-purpose mice (6.26 and 8.82 versus 1.37; $P < 0.001$). In BALB/c mice, only Vi–LT-B elicited higher levels of antibodies than Vi alone (3.62 versus 1.03; $P = 0.0009$); both conjugates elicited higher levels of Vi antibodies after the second injection (6.87 and 17.1 versus 1.31; $P = 0.0001$).

Vi-rEPA elicited significantly higher levels of Vi antibodies than did Vi–LT-B after the second injection in general-purpose mice (59.7 versus 12.3; $P = 0.0001$) and BALB/c mice (17.1 versus 6.87; $P = 0.04$).

Serum Vi antibodies in guinea pigs (Table 3). Guinea pigs, in contrast to mice, do not respond with serum antibodies following injection of Vi (26). Statistical analyses of these data were not performed because the sera from each bleeding were pooled. Vi–LT-B elicited low levels of Vi antibodies after the second injection. Vi-rEPA elicited rises in Vi antibodies after the first (31 versus <2) and second (384 versus 31) injections. Following the third injection of V-rEPA, the Vi antibody level fell from 384 to 167.

Clinical reactions. None of the volunteers had fever following either the first or second injection. Local reactions were confined to mild pain and erythema <2.5 cm in ~25% of the vaccinees after the first injection and in ~4% after the second injection. The serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase levels of 7 days after the first injection were within normal limits in all recipients of each of the three vaccines.

Vi antibodies in the serum of volunteers (Table 4). The preimmunization levels of Vi antibodies were similar in the three groups. As predicted, Vi elicited serum antibodies in

TABLE 4. Vi antibody levels in sera of adult volunteers injected once with 25 μg of Vi alone or two times with 15 μg of Vi as a conjugate

Vaccine injected (lot no.)	No. of volunteers	Mean (range) GM antibody concn (μg/ml; centiles 25–75)[a]			
		Preimmune	Post 1st injection[b]	Post 2nd injection[c]	26 wks post 1st injection
Vi (104a)	25	0.21 (0.14–0.34)	1.77 (0.82–4.11)	ND[d]	0.54 (0.21–2.51)
Vi–LT-B (50860)	25	0.28 (0.21–0.41)	4.74 (1.78–11.5)	4.10 (1.63–13.8)	2.32 (0.72–5.36)
Vi-rEPA (51706)	26	0.21 (0.15–0.36)	4.91 (2.09–8.68)	6.16 (2.29–18.0)	2.69 (1.06–6.02)

[a] 1.77 versus 0.21, 4.74 versus 0.28, and 4.91 versus 0.21, $P < 0.0001$; 4.74 versus 1.77, $P = 0.005$; 4.91 versus 1.77, $P = 0.0006$; 2.32 versus 0.54, $P = 0.04$; 2.69 versus 0.54, $P = 0.01$.

[b] At 4 to 6 weeks after the first injection, a second injection was administered following withdrawal of blood.

[c] Two weeks after the second injection.

[d] ND, not done (no second injection in Vi group).

TABLE 5. Levels of antibodies to LT-B of *E. coli* in sera of adult volunteers injected with Vi alone or as a conjugate

Vaccine (lot no.)	No. of volunteers	GM antibody level (ELISA units)			
		Preimmune	Post 1st injection	Post 2nd injection	26 wks post 2nd injection
Vi	25	167	241	ND[a]	139
Vi–LT-B (50860)	25	249	896	881	295
Vi-rEPA (51706)	26	144	212	141	112

[a] ND, not done.

most of the volunteers (83% had a fourfold or greater rise) (1, 22, 35, 42). After the first immunization, each conjugate elicited higher GM levels of antibodies than did Vi (4.74 versus 1.77, $P = 0.005$; 4.91 versus 1.77, $P = 0.0006$). Of the volunteers injected with Vi-rEPA, 96% had a fourfold or greater rise, and 83% of those injected with Vi–LT-B had a fourfold or greater rise. The levels of Vi antibodies elicited by the conjugates were higher 26 weeks after immunization than elicited by Vi alone (for Vi-rEPA, 2.69 versus 0.54, $P = 0.01$; for Vi-LT-B, 2.32 versus 0.54, $P = 0.04$). As has been observed with other polysaccharide-protein conjugates (6, 7, 31, 35), reinjection of either conjugate into adult volunteers did not elicit a significant rise in antibodies (no booster response). Vi-rEPA elicited higher antibody levels than did Vi–LT-B at each postimmunization interval, but these differences were not significant.

***E. coli* LT-B antibodies (Table 5).** The GM preimmune levels of LT-B antibodies were similar in all groups. There was an approximately fourfold rise in antibodies only in recipients of Vi–LT-B, which did not change after the second injection and which declined to preimmunization levels 26 weeks later.

***P. aeruginosa* ETA antibodies (Table 6).** The GM preimmunization levels of ETA antibodies were similar in all groups. Recipients of Vi-rEPA only had an approximately sixfold rise in ETA antibodies after the first injection, and ETA antibody levels also rose after the second injection (3.58 versus 1.24; $P = 0.04$). These levels declined slightly at 26 weeks (no significant difference) but were still higher than they were prior to immunization (2.78 versus 0.18; $P = 0.0001$).

The failure of Vi–LT-B to elicit a booster response of carrier antibodies, in contrast to Vi-rEPA, is probably due to its higher protein content in a human dose (76.5 μg) compared with Vi–LT-B (10.5 μg).

DISCUSSION

Both Vi–LT-B and Vi-rEPA elicited higher levels of antibodies in mice, guinea pigs, and adult volunteers than did Vi. The antibody levels in volunteers injected with the conjugates, 26 weeks after the first injection, were significantly higher than in the recipients of Vi. Since the main, if not the only, significant effect of capsular polysaccharides is to induce protective levels of serum antibodies, the conjugates can be predicted to be more effective than Vi alone for prevention of typhoid fever (31). Because of its higher immunogenicity in animals and adult volunteers, Vi-rEPA will be used in our first clinical evaluation of the conjugates with children and infants.

The absolute levels of Vi antibodies in serum in this study were slightly lower than those in our previous trials (1, 22, 24, 42). Our best explanation for this difference is that the activity of our Vi-tyramine derivative has declined (25). We are confident, however, that the comparative levels of vaccine-induced Vi antibodies in this study are accurate. The antibody levels in guinea pigs were measured by ELISA with an RIA-calibrated standard. We found that results obtained with ELISA correlated well with those determined by RIA. Furthermore, ELISA could be used for subtype and subclass determination. We plan to use this technique in future studies.

Typhoid fever is considered to be primarily a disease of school age children and young adults and occurs rarely in infants and children <4 years old (28, 29, 44). The reported low incidence in this age group may be due to the limitations of diagnostic procedures. The clinical expression of typhoid fever may be age related (9, 16, 20). Another explanation is that the volume and number of blood samples taken from infants and young children are not optimal for diagnosis of typhoid fever. With the assumption that a positive culture from a bone marrow sample represents 100% of the cases, at least two 5-ml blood samples delivered into selective media are required to achieve an optimal yield of *S. typhi* in patients (17, 19, 21, 23, 27). In Nepal, many children ≤5 years old had serum Vi antibodies (1, 44), suggesting that typhoid fever occurs in younger individuals. In southeast Asia, the incidences of typhoid fever in infants, children, and young adults are similar (19, 36). Control of typhoid fever should, therefore, include vaccination of infants and children in addition to older age groups. Typhoid immunization would be facilitated if it could be included in the routine schedule of infants in countries where this disease is endemic. On the basis of our experience with *Haemophilus influenzae* type b and pneumococcus type 6A polysaccharide conjugates (6, 34, 35), it is likely that these Vi conjugates will elicit protective levels of antibodies in infants and children (31).

TABLE 6. Levels of antibodies to *P. aeruginosa* ETA in sera of adult volunteers injected with Vi alone or as a conjugate

Vaccine (lot no.)	No. of volunteers	GM antibody level (ELISA units)[a]			
		Preimmune	Post 1st injection	Post 2nd injection	26 wks post 2nd injection
Vi	25	0.20	0.22	ND[b]	0.10
Vi–LT-B (50860)	25	0.20	0.27	0.23	0.16
Vi-rEPA (51706)	26	0.18	1.24	3.58	2.78

[a] 3.58 and 2.78 versus 0.18, $P = < 0.001$; 3.58 versus 1.24, $P = 0.04$.
[b] ND, not done.

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